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(54) Title: PRODUCTION OF ORGANIC COMPOUNDS

(57) Abstract

The present invention relates to the production of organic compounds and in particular to the production of isotopically labelled organic compounds. The present invention provides a process for the biological photoproduction of a labelled organic compound comprising the steps of culturing, in a culture medium containing at least one simple labelled inorganic nutrient, a mutant strain of photosynthetic prokaryotic or eukaryotic species selected from cyanobacterial or microalgal species which are capable of producing a said organic compound, and recovering labelled organic compound produced by the mutant strain from the culture medium. Thus with the present invention it is possible to achieve continuous production of isotopically labelled organic compound over an extended period of time using relatively simple and hence inexpensive feedstuffs.

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PRODUCTION OF ORGANIC COMPOUNDS

The present invention relates to the production of organic compounds and in particular to the production of isotopically labelled organic compounds.

Previously known processes for the production of isotopically labelled organic compounds have generally relied on the use of synthetic organic chemistry processes which tend to be very expensive due to the relatively small scale of production involved. More recently, processes have been used in which biological cells are fed with suitable labelled precursors following which the cells are broken up and the desired organic compound recovered from the resulting mixture. However, on the one hand, such processes require the use of relatively complex and expensive precursors and, on the other hand, the need for destruction of the cells and subsequent fractionation procedures is rather inefficient and results in further expense. It has also been suggested that labelled organic compounds could be produced by exotic biological cells under anaerobic conditions using suitably labelled feedstuffs. On the one hand, the use of anaerobic conditions involves significant additional costs and difficulties in maintaining the necessary anaerobic environment and on the other hand anaerobic bacteria have further special requirements. More particularly, whilst aerobic bacteria simply utilise water as an electron donor, anaerobic bacteria require expensive and dangerous materials such as hydrogen and hydrogen sulphide as electron donors.

It is an object of the present invention to avoid or minimise one or more of the above disadvantages.

The present invention provides a process for

the biological photoproduction of labelled organic compound comprising the steps of culturing under aerobic conditions, in a culture medium containing at least one simple labelled inorganic nutrient, a mutant strain of photosynthetic prokaryotic or eukaryotic species selected from cyanobacterial or microalgal species which are capable of producing a said organic compound under aerobic conditions, and recovering labelled organic compound produced by said mutant strain from said culture medium.

In a further aspect the present invention provides a process for biological photoproduction of labelled organic compound comprising the steps of subjecting an aerobic strain, selected from prokaryotic cyanobacterial and eukaryotic microalgal species, to mutagenise so as to produce a plurality of mutant strains of said species, selecting a mutant strain capable of producing a said organic compound from said plurality by culture thereof with an analogue, as defined hereinbelow, of said organic compound so as to inhibit the other strains in said plurality, culturing said mutant strain in a culture medium with a supply of a simple labelled inorganic nutrient, and recovering labelled organic compound produced by said mutant strain from said culture medium.

As used herein the term "analogue" is used in relation to the desired organic compound to indicate another organic compound, usually synthetic, of more or less closely analogous structure to that of the desired organic compound and to which certain mutant strains capable of over-producing the desired organic compound and liberating elevated levels of said desired organic compound into a medium, are resistant whilst other strains are not so resistant (see for example W. Phares et al J. Bacteriol 122 (3) 943 (1975) and G. Riccardi et al J. Bacteriol 147 1002 (1981)).

Thus with a process of the present invention it is possible to achieve continuous production of isotopically labelled organic compound over an extended period of time using relatively simple and hence inexpensive nutrients or feedstuffs.

Suitable simple inorganic nutrients or feedstuffs that may be used in accordance with the present invention include nitrogen gas, ammonia and nitrate in which the nitrogen atoms are labelled and carbon dioxide and bicarbonate in which the carbon atoms are labelled. The labelled atoms may be radioactive isotopes such as ^{14}C Advantageously, though, non-radioactive isotopes such as ^{15}N and ^{13}C can be used.

In order to facilitate recovery of the labelled organic compounds from the cell culture medium, the cells of the mutant strain are advantageously immobilised in, for example natural polymers such as calcium alginate, agar and carrageenan or synthetic polymers such as polyurethane or polyvinyl foams. This also has the further advantage that the cells grow more slowly or stop growing with the result that a better yield of the desired organic compound can be obtained. Yet another advantage is that the immobilised cells are significantly more resistant to environmental shock e.g. substantial pH change, than they would otherwise be. It is also possible to employ a continuous culture system, in which nutrients enter and leave the fermenter.

In order to increase still further the release of the desired organic compound from the cells into the medium, without significantly affecting their viability, the cells may be subjected to osmotic shock,

for example, by acclimatising them to an elevated ionic strength medium such as BGS-11 containing added 490 mol m^{-3} sodium chloride and 10 mol m^{-3} calcium chloride (as described in R.H. Reed et al in Enzyme Microb. Technol. 8 101 (1986)) and then transferring them to similar medium (BG-11) without the added salts. Alternatively the cells could be exposed to detergents such as polyoxyethylene stearate detergents especially MYRJ 45 and MYRJ 52 available from Serva Feinbiochemica of Heidelberg, West Germany

at low levels of the order of 0.1 to 1% w/v, preferably 0.5% w/v, as described in G.W. Niven et al in J. General Microbiol 134 689 (1988).

The mutant cells in accordance with the present invention are obtained more or less readily and significant numbers of useful strains may be obtained with each mutagenesis procedure. Furthermore the mutant strains may be readily cultured and have good stability over extended periods of time (e.g. 2 months or more in continuous culture) without reversion to wild type. They are also relatively long lived and can continue overproducing labelled organic compound(s) for 2 months or more. Nevertheless it is also possible, if desired, to recover labelled organic compounds from inside the cells by disruption thereof by means of well known cell lysis procedures.

Various organic compounds can be produced by the process of the invention including in particular various amino acids such as alanine, methionine, phenylalanine, tyrosine, tryptophan, leucine and isoleucine, amino acid derivatives such as glycine betaine and methylglutamine and carbohydrates, especially sugars such as sucrose and glucose.

As noted above, various labelled organic compounds may be produced by the process of the present invention.

In particular the following compounds may be produced by the process of the invention and the invention extends to these compounds when produced by the process of the invention:

Phenylalanine (especially $C_6H_5CH_2CH(^{15}NH_2)CO_2H$)
Methionine (especially CH_3S, CH_2, CH_2, CH $(^{15}NH_2)$, CO_2H)
Sucrose (i.e. fructose + glucose):
fructose (especially $^{13}CH_2OH$, $^{13}C{=}O$, $^{13}CH(OH)$, $^{13}CH(OH)$, $^{13}CH(OH)$, $^{13}CH_2(OH)$,
glucose
($H^{13}C{=}O$, $^{13}CH(OH)$, $^{13}CH(OH)$, $^{13}CH(OH)$, $^{13}CH(OH)$, $^{13}CH_2(OH)$

The desired organic compounds may be recovered from the cell support media or lysate mixture, by any suitable technique known in the art for the separation and isolation of such compounds. Conveniently though there are used in the case of continuous culture systems, continuous in-line techniques which do not substantially interfere with the further production of the desired organic compound by the cells. Thus there may be used for example a column containing an ion exchange resin which will selectively retain amino acids from culture medium cycled therethrough. The trapped amino acids may subsequently be fractionated and recovered from the column by elution with changes of pH and/or ionic strength.

By means of mutagenesis of known microalgal and cyanobacterial strains and selection of a resulting mutant it is readily possible to obtain individual

strains producing specific organic compounds or groups of organic compounds. Suitable mutagenic processes and mutant selection on the basis of exposure to analogues of the desired organic compound are well known in the art (see for example K. Yamada et al. The Microbial Production of Amino Acids. John Wiley and Sons. New York (1972)).

In general, mutagenising conditions are selected so as to give a very high rate of kill so that a high proportion of the surviving strains have significant mutations. Thus for example there may be used N-methyl-N'-nitro-N-nitrosoguanidine at a concentration of the order of 250 μg ml^{-1} under fluorescent lighting for a time required to give a kill of the order of 99% of the original cells.

As noted above the mutant strain cells used in accordance with the invention have good stability. Nevertheless, if desired, they could be periodically subjected to further exposure to the appropriate analogue to inhibit any possible reversion to wild type which could result in reduced yield and/or increased production of undesired compounds.

It will also be appreciated that various methods may be used for culturing the mutant strain cells which may be immobilised in suitable polymeric substrate as described above or freely suspended in the medium using any suitable culture conditions. In general though there is preferably used for cyanobacterial mutant strains a temperature of from 18 to 30°C, a pH of from 7.5 to 11.0, and a photon flux density of from 20 to 500 $\mu E.\ m^{-2}\ s^{-1}$.

Any suitable cyanobacterial or microalgal species may be used in accordance with the present invention. Particular species that may be mentioned are Anabaena

variabilis as deposited in the American Type Culture Collection under accession No. ATCC 29413 and Synechocystis as deposited in the Pasteur Institute Culture Collection under the accession No. PCC 6803.

Whilst the present invention is particularly concerned with the production of organic compounds labelled with carbon and nitrogen isotopes it also extends to the use of isotopes of other atoms such as sulphur in particular ^{35}S. Suitable inorganic nutrients that may be used include labelled sulphate (e.g. $Fe^{35}SO_4$). Labelled organic compounds that may be produced by using these include labelled cysteine and methionine.

Further preferred features and advantages of the present invention will appear from the examples given by way of illustration only.

Example 1 - Production of labelled amino acids

A. Preparation of mutagenised cells

Species of nitrogen-fixing cyanobacteria, such as Anabaena spp. grown either in nitrogen-free or nitrate containing medium were harvested by centrifugation and resuspended in 10mM HEPES/NaOH buffer, pH 7.0. Cultures were sonicated to break the filaments to an average length of 2 cells and washed twice in the above buffer and 250 $\mu g\ ml^{-1}$ N-methyl-N^1-nitro-N nitrosoguanidine.

The suspension was incubated for 2 hrs at 30°C in fluorescent lighting with shaking. The amount of mutagen or time of mutagenesis may be varied. The treatment was terminated by washing the cells three times with growth media such as BG11 or $BG11_0$ (see Rippka et al J. Gen Microbiol 111 1-61 (1979)). This treatment results in approximately 1% survival of cells.

In order to isolate mutant strains resistant to amino

acid analogues, mutagenised cells were grown for 7 days at 30°C at a photon fluence rate of 60μE.$m^{-2}.s^{-1}$ and then following sonication to break filaments to an average length of two cells plated onto BG 11_0 plates solidified with 1% (w/v) agar containing 10mM HEPES, pH 8.0 and either 40 μg. ml^{-1} 6-fluorotryptophan or 60 μg. ml^{-1} ethionine. Colonies resistant to amino acid analogues were picked after 10-14 days incubation in the light of 30°C and were restreaked on selective medium a further five times prior to growth in liquid $BG11_0$ containing 10mM HEPES, pH 8.0 and either 40 μg.ml^{-1} fluorotryptophan or 60 μg. ml^{-1} ethionine.

B. Immobilisation of mutagenised cells

Cells were harvested from exponential phase cultures by centrifugation and the pellet was resuspended in a 2% (w/v) aqueous sterile solution of sodium alginate This mixture was pumped dropwise into a sterile 0.1M Cl_2 solution in a liminar flow cabinet using a multichannel peristalic pump.

C. Production of labelled amino-acids

The beads thus formed were removed after 30 min and loaded into bioreactors such as air-lift gas reactors with gas recycling. A suitable initial chlorophyll a concentration of the beads was 30 μg. (g wet weight) $^{-1}$. Such reactors were maintained in the light (80-200μE. $m^{-2}s^{-1}$) at 25-30°C, bubbled with air and supplied with a medium such as BG11 buffered to pH 9.0 containing ^{15}N labelled nitrate and ^{13}C labelled bicarbonate either together or singly to produce either doubly or singly labelled amino acids. The labelled amino acids produced by the mutagenised cells are released into the medium. To maximise the incorportation of label from substrate to product, fed-batch cultures may be employed. Amino acids such as alanine, phenylalanine, tryptophan,

isoleucine and leucine are released by mutants resistant to 6-fluorotryptophan and do not require extraction or separation from the cells which remain immobilised.

Increased yields of released amino acids may be achieved by the addition of surfactants or by osmotic shock.

Following a production run, cells may be harvested and hydrolysed with acid (6N-HCl) to extract from the cells of nitrogen - fixing species and non-nitrogen species of prokaryotic and eukaryotic microorganisms for the production of amino acids as outlined above as well as of sugars, which are photosynthetically generated.

With the above procedures it has been found possible to obtain yields of up to 200 mg of labelled amino acid per litre of medium in batch culture using a 6-fluorotryptophan resistant mutant strain FT2 of Anabaena variabilis (ATCC 29413) and in continuous culture with the same strain yields of up to 50 mg of labelled amino acid have been obtained at a dilution rate of 0.007 h^{-1} with continuous labelled amino acid production extending for over 8 weeks.

Example 2 - Production of ^{14}C radioactively labelled amino acids

Cells of the abovementioned FT2 mutant strain were suspended in the abovedescribed BG11 culture medium at a chlorophyll a concentration of 4.34 µg ml^{-1} buffered with Tricine (10mM final concentration) to pH 8.0. Cells were conditioned in the light (65µE. m^{-2}, s^{-1}) for 30 min at room temperature (20°C) prior to the addition of 50 mM $NaH^{14}CO_3$ (specific activity 0.2 µCi µmol^{-1}). The rate of $^{14}CO_2$ fixation was 32.8 µmol. h^{-1} mg chl a^{-1} which was sustained throughout the experimental period of (120 h). Following this

period, cells were separated from the incubation medium by centrifugation (12000 x g, 5 min) and ^{14}C- labelled HCO^-_3 was removed by acidification to the medium with HCl (final concentration 2M). A large percentage of the $H^{14}CO^-_3$ (60%) which had previously been fixed by the cells reappeared in the medium in an acid stable form. Of the total radioactivity taken up by the cells, 16% was released into the medium as ^{14}C- labelled phenylalanine. ^{14}C-labelled amino acids liberated into the medium were separated by reverse phase HPLC following derivatisation with PITC (phenylisothiocyanate).

Example- Production of labelled amino acids

Table 1 provides an indication of relative amounts of a number of different amino acids produced by various 6-fluorotryptophan-resistant mutant strains of Anabaena variabilis (FT) and of Synechocystis 6803 (FTS) using labelled nitrate feedstuffs for the latter (FTS) mutants and nitrogen (gas) in air for the former (FT) mutants. The yields are for cells after 14 days growth in batch culture and only those amino acids released into the medium at a rate greater than 1 μmol (mg chlorophyll a)$^{-1}$ are indicated.

Table 1

Amino acid liberation by 6-FT-resistant mutant strains in μmol (mg chla)$^{-1}$

	Anabaena variabilis mutants			Synechocystis 6803 mutants		
Amino acid	FT-2	FT-7	FT-9	FTS-4	FTS-11	FTS-42
Ser	-	-	-	1.3	2.9	6.6
Gly	-	-	-	1.3	-	6.2
Ala	1.3	12.2	9.3	31.7	-	70.1
Val	-	-	-	-	-	3.7
Ile	-	1.3	-	6.1	2.8	4.2
Leu	-	1.0	1.6	2.6	-	3.6
Tyr	1.6	-	-	-	-	1.8
Phe	6.4	1.1	-	1.1	-	1.6
Orn	-	-	-	-	-	2.3
Total	13.0	20.4	16.2	56.3	14.4	110.4

Thus as may be seen from the above table mutant FT-2 is particularly suitable for producing labelled phenylalanine, whilst FT-7 and FT-9 are particularly suitable for producing labelled alanine.

CLAIMS

1. A process for the biological photoproduction of a labelled organic compound comprising the steps of culturing under aerobic conditions, in a culture medium containing at least one simple labelled inorganic nutrient, a mutant strain of photosynthetic prokaryotic or eukaroytic species selected from cyanobacterial or microalgal species which are capable of producing a said organic compound under aerobic conditions, and recovering labelled organic compound produced by said mutant strain from said culture medium.

2. A process according to claim 1 wherein is selected a mutant strain capable of producing an amino acid.

3. A process according to claim 1 wherein is selected a mutant strain capable of producing a sugar.

4. A process according to claim 2 wherein is used at least one inorganic nutrient selected from nitrogen gas, ammonia, and a nitrate in which are present isotopically labelled nitrogen atoms, and carbon dioxide and bicarbonate in which are present isotopically labelled carbon atoms.

5. A process according to claim 3 wherein is used at least one inorganic nutrient selected from carbon dioxide and bicarbonate in which are present isotopically labelled carbon atoms.

6. A process according to claim 4 wherein is used a nutrient in which are present ^{15}N atoms.

7. A process according to claim 4 or claim 5 wherein is used a nutrient in which are present ^{14}C or ^{13}C atoms.

8. A process according any one of claims 1 to 7 wherein the cells of the mutant strain are immobilised in polymeric substrates.

9. A process as claimed in claim 8 wherein the cells of the mutant strain are immobilised in beads of

natural polymer selected from calcium alginate, agar and carrageenan.

10. A process as claimed in claim 8 wherein the cells of the mutant strain are immobilised in particles of synthetic polymer selected from polyurethane foam and polyvinyl foam.

11. A process as claimed in any one of claims 1 to 10 which includes the step of subjecting the cells of said mutant strain to osmotic shock so as to increase release of labelled organic compound into the culture medium without killing said cells.

12. A process as claimed in any one of claims 1 to 11 wherein a detergent is introduced into the medium so as to increase release of labelled organic compound into the culture medium.

13. A process as claimed in any one of claims 1 to 12 wherein the mutant strain is cultured at a temperature of from 18 to 30°C, at pH in the range from 7.5 to 11, and under a photon flux density of from 20 to 500 μE. $m^{-2}s^{-1}$.

14. A process as claimed in any one of claims 1 to 13 which includes the preliminary step of subjecting a wild strain of said photosynthetic prokaryotic or eukaryotic species to mutagensis so as to produce a plurality of mutant strains of said species, selecting a mutant strain capable of producing a said organic compound from said plurality of mutant strains, by culture thereof with an analogue, as defined herein-before, of said organic compound, so as to inhibit the other strains in said plurality, and recovering said selected mutant strain.

15. A process for the biological photoproduction of a labelled organic compound comprising the steps of subjecting an aerobic strain, selected from prokaryotic cyanobacterial and eukaryotic microalgal species, to

mutagenesis so as to produce a plurality of mutant strains of said species, selecting a mutant strain capable of producing a said organic compound from said plurality by culture thereof with an analogue, as defined hereinbelow, of said organic compound so as to inhibit the other strains in said plurality, culturing said mutant strain in a culture medium with a supply of a simple labelled inorganic nutrient under aerobic conditions, and recovering labelled organic compound produced by said mutant strain from said culture medium.

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 88/00510

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[4]: C 12 P 1/00; C 07 B 59/00

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[4]	C 12 P; C 07 B

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with Indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	Chemical Abstracts, volume 107, no. 1, 6 July 1987, (Columbus, Ohio, US), S.A. Macko et al.: "Isotopic fractionation of nitrogen and carbon in the synthesis of amino acids by microorganisms", see page 369, abstract 3983m, & Chem. Geol. 1987, 65(1), 79-92	1,2,4,6, 14,15
A	EP, A, 0220951 (CELANESE CORP.) 6 May 1987 see claims	1

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* Special categories of cited documents: [10]

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
23rd September 1988	17 OCT 1988
International Searching Authority	Signature of Authorized Officer
EUROPEAN PATENT OFFICE	P.C.G. VAN DER PUTTEN

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. GB 8800510 SA 22978

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 03/10/88
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0220951	06-05-87	JP-A- 62130692	12-06-87